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Mechanism of Action of Sparfloxacin against and Mechanism of Resistance in Gram-Negative and Gram-Positive Bacteria

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The inhibition of DNA synthesis by sparfloxacin; accumulation of sparfloxacin into members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and staphylococci; induction of *recA* in *Escherichia coli*; and the optimum bactericidal concentration (OBC) were measured, and killing kinetics at the OBC were estimated. The OBC and maximum *recA*-inducing concentration in *E. coli* were both 1 μg of sparfloxacin per ml. Accumulation was rapid; two- to threefold more sparfloxacin than ciprofloxacin accumulated in staphylococci and more sparfloxacin accumulated in staphylococci than in gram-negative bacteria. Laboratory mutants with decreased susceptibilities to quinolones alone or multiply resistant were selected from the *Enterobacteriaceae* and *Staphylococcus aureus* by using sparfloxacin.

Sparfloxacin is a new difluorinated quinolone with similar activity for gram-negative and gram-positive bacteria and a spectrum of activity that includes anaerobes, *Chlamydia trachomatis*, *Mycoplasma* spp., and mycobacteria (3, 4, 7, 16, 24). As with some other quinolones, the activity of sparfloxacin is reduced at acid pH and in the presence of cations (e.g., 9 mM Mg^{2+} or 4 mM Ca^{2+}; 4). Laboratory mutants of members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Xanthomonas maltophilia*, and *Staphylococcus aureus* with progressive resistance to sparfloxacin were selected by repeated subculture over 15 days, but the mechanism was not described (4). In this study, the action of sparfloxacin on *Enterobacteriaceae*, *P. aeruginosa*, and staphylococci was investigated. In addition, laboratory mutants with decreased susceptibilities to each quinolone were selected, and their phenotypes were determined.

The optimum bactericidal concentration (OBC) of sparfloxacin and the rate of killing at the OBC were determined for the NCTC type strains of *Escherichia coli*, *Enterobacter cloacae*, *Serratia marcescens*, *Klebsiella pneumoniae*, *P. aeruginosa*, *S. aureus*, and *Staphylococcus epidermidis* (19). Induction of the SOS response was measured by determining the increase in *recA* expression in *E. coli* GC2241 containing a gene fusion between *recA* and *lac* (22). The inhibition of DNA synthesis in the *Enterobacteriaceae* and staphylococci by sparfloxacin was determined by measuring the incorporation of [^{3}H]thymidine into DNA (25), and in *P. aeruginosa*, the incorporation of [^{3}H]adenine was measured (1). The accumulation of sparfloxacin was measured by using a modification of the method of N. Moreau (personal communication). Mid-exponential-phase bacteria were washed and suspended to 1/20 the original volume in phosphate-buffered saline at 4°C and kept on ice. [^{14}C]sparfloxacin (10 μg/ml; specific activity, 21.4 μCi/mg; Rhone Poulenc D.P.C) was added to 8 ml of cell suspension that had equilibrated at 37°C for 10 min. At timed intervals, 500-μl samples were withdrawn, placed in duplicate tubes containing 500 μl of silicon oil (six parts 550:5 parts 556) on ice, and centrifuged immediately at 13,000 × *g* for 90 s, and the top (aqueous) layer was removed. The pellets were snap frozen in a methanol–dry-ice bath and then stored at −20°C. The oil layer was removed, the pellets were resuspended, and the activity was determined by scintillation counting. Binding to the cell surface was estimated by measuring accumulation at 0°C. The modified fluorometric method was used for measuring ciprofloxacin and norfloxacin accumulations (15). The accumulation data from each procedure were converted and expressed as nanograms of quinolone per milligram (dry weight) of cells.

Mutants with decreased susceptibilities to sparfloxacin were selected from the NCTC type strains of *E. coli*, *E. cloacae*, *S. marcescens*, *K. pneumoniae*, and *S. aureus* on agar containing 3, 5, and 10 times the MIC of the drug. Putative mutants from each selection condition were examined for susceptibility to the agents listed in Table 3 by the agar doubling-dilution method. The kinetics of growth of all mutants was examined by measuring the optical density of the growing culture at timed intervals at 675 nm, and the biochemical properties of all mutants were examined by using the API 20E (API Laboratory Products, Basingstoke, United Kingdom) system. The outer membrane proteins (OMPs) of all strains were prepared by using differential centrifugation, sonication, and Sarkosyl extraction (20). All samples were electrophoresed on two systems, 10 and 14% vertical sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 20 μg of protein per channel. The inhibition of DNA synthesis and accumulation of quinolones in the mutants were examined as described above. *E. coli* S17-1 containing plasmid pNJR3-2, which contains quinolone-susceptible *E. coli gyrA*, was conjugated with selected mutants by the protocol of Robillard (23).

The NCTC type strain of each species was inhibited by sparfloxacin at concentrations typical of a susceptible strain, being most active for *E. coli* and least active for *P. aeruginosa* (Table 1). The MIC of sparfloxacin was similar to that of ciprofloxacin for *S. aureus*. The OBC was 10- to 60-fold higher than the MIC in all strains (Table 1). At the OBC of sparfloxacin for *E. coli*, *E. cloacae*, and *P. aeruginosa*, there was a similar rate of kill such that after 1 h of exposure, the number of viable bacteria remaining (from an initial inoculum of ~10^8 CFU/ml) had decreased by 5 × 10^4 to 5 × 10^5 CFU/ml. At the OBC of sparfloxacin for *S. marcescens*, *K. pneumoniae*, *S. aureus*, and *S. epidermidis*, there was a

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TABLE 1. Susceptibility, bactericidal activity, and inhibition of DNA synthesis by sparfloxacin and ciprofloxacin

Species and strain[a]	Sparfloxacin				Ciprofloxacin			
	MIC (μg/ml)	OBC[b]	Decrease in viable count[c]	IC_{50} (μg/ml)	MIC (μg/ml)	OBC	Decrease in viable count	IC_{50} (μg/ml)
E. coli AB1157	0.015	1	4.5	0.043	0.008	1	4.5	0.011
E. coli KL-16	0.015	5	4.5	0.095	0.008	1	4.5	0.015
E. coli NCTC 10538	0.03	1	5	0.026	0.015	3	5	0.015
E. cloacae NCTC 10005	0.06	5	5	0.0125	0.008	1	4.5	0.0038
S. marcescens NCTC 10211	0.5	100	5	0.47	0.06	3	3	0.058
K. pneumoniae NCTC 9633	0.03	10	6	0.046	0.03	3	4.5	0.026
P. aeruginosa NCTC 10662	2	100	5	2	0.25	10	4.8	0.34
S. aureus NCTC 8532	0.12	10	3	0.058	0.25	10	2	0.20
S. epidermidis NCTC 11047	0.12	10	3	0.295	0.12	3	2	>2

[a] 10^6 CFU of each strain.
[b] After 1 h.
[c] Log_{10} decrease at OBC.

greater drop in the viable count than with ciprofloxacin. Despite the low MIC of sparfloxacin for *S. marcescens*, the OBC was reproducibly high. The concentration of sparfloxacin that inhibited DNA synthesis by 50% (IC_{50}) correlated well with the MIC (correlation coefficient = 0.98). After 40 min of exposure, *E. coli* GC2241 (a derivative of AB1157) maximally expressed *recA* at 1 μg of sparfloxacin per ml, although induction was detected at 0.01 μg/ml. The maximum inducing concentration correlated well with the OBC of *E. coli* AB1157.

The accumulation of sparfloxacin was measured by three methods: (i) the fluorometric method (2, 15), which uses the natural fluorescence of the fluoroquinolone molecule for detection (however, sparfloxacin, despite being difluorinated, fluoresced poorly, such that even with 50 μg/ml, unreliable data were obtained for all strains); (ii) a vacuum filtration method (8) (however, the [^{14}C]sparfloxacin bound to all brands of filter tested, so that any accumulation was masked); and (iii) measurement of the accumulation of [^{14}C]sparfloxacin in all strains by partitioning of the cells (after exposure to drug) in silicon oil, centrifugation, and scintillation counting. All strains except *P. aeruginosa* rapidly accumulated sparfloxacin, with high concentrations accumulated by the staphylococci (Table 2, Fig. 1). The steady-state concentration was reduced threefold by the presence of 7 mM magnesium chloride. Ciprofloxacin took longer than sparfloxacin to reach steady state in the *Enterobacteriaceae* but achieved a higher concentration.

Sparfloxacin was less active than ciprofloxacin for quinolone-resistant mutants of four species of *Enterobacteriaceae* selected in a previous study (18), particularly for mutant *S. marcescens* (Table 3). There was a 4- to 32-fold difference between the activity of sparfloxacin for bacteria expressing a *gyrA* phenotype (resistant to quinolones alone) and that for wild type, but between the multiple-resistance phenotype and the *gyrA* phenotype, there was only a 2-fold difference. Sparfloxacin selected mutants of *Enterobacteriaceae* and *S. aureus* with decreased susceptibility at a frequency of mutation to resistance similar to that for ciprofloxacin (10^{-7} to 10^{-10}) except for *E. cloacae* NCTC 10005, which was more difficult to select with sparfloxacin (frequency of mutation, 10^{-10} to 10^{-11}). Different patterns of cross-resistance to antibiotics other than quinolones were shown by the different species (Table 3). Multiply resistant *E. coli* was resistant to trimethoprim, and one mutant was also resistant to cefoxitin. Multiply resistant *E. cloacae* was resistant to chloramphenicol, tetracycline, and trimethoprim. Multiply resistant *K. pneumoniae* was resistant to chloramphenicol, cefoxitin, and trimethoprim; two mutants were resistant to chloramphenicol and trimethoprim; and one mutant was resistant to chloramphenicol. One multiply resistant mutant of *S. aureus* was resistant to chloramphenicol, and three mutants were resistant to trimethoprim. None of the mutants had altered API 20E profiles, and only one *S. marcescens* strain (B15S2) grew slowly (data not shown). Only the mutants of *S. marcescens* had the altered OMP profiles usually associated with multiple cross-resistance. Surprisingly, four sparfloxacin-selected *S. aureus* isolates were cross-resistant, but no difference in the protein profile of the whole-cell lysate from that of the wild-type parental strain was observed (data not shown). A greater decrease in the activity of ciprofloxacin than in that of sparfloxacin was

TABLE 2. Accumulation of sparfloxacin and ciprofloxacin at 10 μg/ml

Species and strain	Sparfloxacin		Ciprofloxacin	
	SSC[a] (ng/mg)	Time to steady state (s)	SSC (ng/mg)	Time to steady state (s)
E. coli NCTC 10538	53	60	60	60
E. cloacae NCTC 10005	50	30	75	56
S. marcescens NCTC 10211	7	40[b]	56	65
K. pneumoniae NCTC 9633	29	40	28	50
P. aeruginosa NCTC 10662	10	150[b]	35	90
S. aureus NCTC 8532	150	60	54	60
S. epidermidis NCTC 11047	180	60	70	300

[a] SSC, steady-state concentration (mean from the data of at least three experiments).
[b] Estimate, since the accumulation was low.

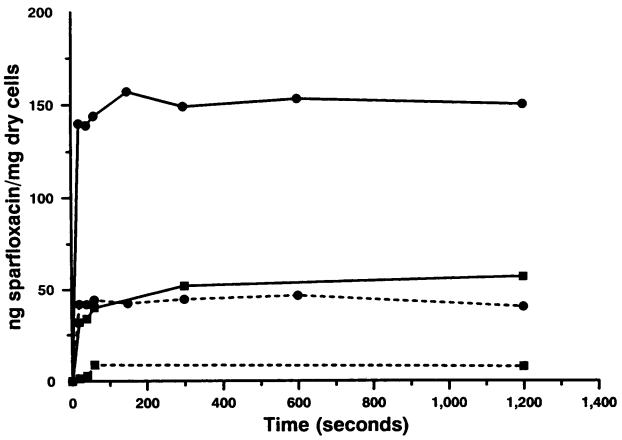

FIG. 1. Accumulation of sparfloxacin by *E. coli* NCTC 10538 (■) and *S. aureus* NCTC 8532 (●). Dotted lines show accumulation in the presence of 7 mM $MgCl_2$.

seen for the mutant *S. aureus*; one *S. aureus* mutant (F77C4) became fluoroquinolone resistant but more susceptible to nalidixic acid (MIC, 16 μg/ml).

The IC_{50}s of sparfloxacin and ciprofloxacin for DNA synthesis in most ciprofloxacin-selected mutants were increased 10- to 100-fold and correlated with the MIC (Table 3), whereas there was no change in the accumulation of ciprofloxacin. Four of the sparfloxacin-selected *E. coli* mutants were cross-resistant, had decreased accumulation of norfloxacin (insufficient [^{14}C]sparfloxacin to use with the mutant bacteria), and no change in the IC_{50}. The *E. cloacae* mutant had a raised IC_{50}; those selected with ciprofloxacin had no change in the accumulation of ciprofloxacin, but the sparfloxacin-selected mutants accumulated less norfloxacin. Of particular interest were the mutant *S. marcescens*, which had several phenotypes on 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis: wild type, decrease in 39-kDa OMP, or decrease in 39-kDa OMP with increase or decrease in 41-kDa OMP. Some mutants also had a decreased quinolone accumulation plus an increased IC_{50}. Mutant *K. pneumoniae* also had an increased IC_{50} and decreased quinolone accumulation compared with wild type. Mutant *S. aureus* had an increased IC_{50} and no change in accumulation of quinolones.

E. coli S17-1 containing plasmid pNJR3-2 containing *gyrA* susceptible to quinolones was mated with all the mutants listed in Table 3; only the mating with mutant *K. pneumoniae* yielded transconjugants. No spontaneous tetracycline-resistant *K. pneumoniae* was selected. All 20 transconjugants screened were highly resistant to tetracycline, became

TABLE 3. Phenotypes of typical ciprofloxacin- and sparfloxacin-resistant mutants[a]

Strain	Selecting agent[b]	MIC (μg/ml)					IC_{50} (μg/ml) of DNA synthesis		Uptake (μg/ml)[c]	
		SPAR	CIP	NAL	CHLOR	TMP	SPAR	CIP	CIP	NOR
E. coli										
NCTC 10538, I114		0.03	0.015	4	8	0.5	0.043	0.011	58	102
gyrA, I201[d]		0.12	0.06	256	4	0.5				
Multiply resistant, I202[d]		0.12	0.015	16	16	2				
I114C7	CIP × 10	0.12	0.12	>128	8	1	0.58	0.7	59	
I114S2	SPA × 3	0.12	0.25	64	64	16	0.04	0.05		59
E. cloacae										
A1, WT		0.03	0.008	4	2	0.5	0.033	0.0038	70	108
gyrA, A76[d]		0.5	0.25	128	4	1				
Multiply resistant, A77[d]		0.25	0.25	32	64	32				
A1C1	CIP × 3	1	0.25	64	64	16	0.85	0.34	75	
A1S1	SPAR × 3	0.5	0.5	64	64	16	0.34	0.04		80
S. marcescens										
B15, WT		0.5	0.06	8	16	8	0.47	0.058	55	62
gyrA, B53[d]		4	0.5	128	8	8				
Multiply resistant, B54[d]		2	0.25	32	128	32				
B15C1	CIP × 3	4	0.5	64	>128	32	1	0.78	47	
B15S2	SPAR × 3	2	1	64	>128	32	1.2	0.62	5	39
K. pneumoniae										
H43, WT		0.03	0.03	8	4	2	0.046	0.026	36	52
gyrA, H113[d]		1	1	512	4	2				
H43C1	CIP × 3	0.5	0.5	128	128	32	0.85	0.95	25	
H43SI1	SPAR × 10	0.5	0.5	>128	128	16	0.26	0.31		27
S. aureus										
F77, WT		0.12	0.25	>128	2	0.5	0.03	0.20	62	70
F77C2	CIP × 3	0.25	4	>128	4	0.5	0.85	0.9	51	
F77S2	SPAR × 3	0.5	1	>128	8	4				85

[a] SPAR, sparfloxacin; CIP, ciprofloxacin; NAL, nalidixic acid; CHLOR, chloramphenicol; TMP, trimethoprim; NOR, norfloxacin; WT, wild type.
[b] Multiplier indicates concentration of drug relative to MIC; e.g., CIP × 10 indicates 10 times the MIC of ciprofloxacin.
[c] Steady-state concentration after 5 min of exposure.
[d] Isolated in a previous study (18).

TABLE 4. Susceptibility of recipient *K. pneumoniae* and transconjugants after mating with *E. coli* containing *gyrA* gene probe

Organism	MIC (μg/ml)[a]					
	SPAR	NAL	CIP	CHLOR	TMP	TET
Vector control × H43C1	0.5	128	1	>128	32	16
Recipient H43C1	0.5	128	1	>128	32	16
Transconjugants (H43C1 × PNJR3-2)	0.12	16–32	0.5	16–64	8	>128
Vector control × H43S11	0.5	128	1	>128	32	16
Recipient H43S11	0.5	128	0.5	>128	32	16
Transconjugants (H43S11 × PJR3-2)	0.12	16–32	0.5	16–64	8	>128

[a] SPAR, sparfloxacin; NAL, nalidixic acid; CIP, ciprofloxacin; CHLOR, chloramphenicol; TMP, trimethoprim; TET, tetracycline.

fourfold more susceptible to sparfloxacin and nalidixic acid, but had little change in the MIC of ciprofloxacin (Table 4) compared with changes in the vector control and mutants. Interestingly, the MICs of trimethoprim and chloramphenicol also decreased in the transconjugants.

The susceptibility data for the NCTC type strains obtained in this study compare well with previously published data (3, 4, 7, 24) and confirmed the good antistaphylococcal activity of sparfloxacin. Like other quinolones (5, 14, 21), sparfloxacin inhibited DNA synthesis in the susceptible bacteria at concentrations correlating well with the MIC. The maximum *recA*-inducing concentration was similar to the IC_{50} for DNA synthesis, suggesting that at concentrations of sparfloxacin that inhibit DNA synthesis, there is also damage to DNA (thereby inducing *recA* expression and the SOS response).

The OBC correlated with maximum *recA* expression. At low concentrations, sparfloxacin did not kill *S. marcescens* as rapidly as it killed other bacteria studied, even though *S. marcescens* is susceptible to 0.25 μg/ml, and quinolone-resistant *S. marcescens* had a higher MIC of sparfloxacin than did quinolone-resistant mutants of other *Enterobacteriaceae*. Sparfloxacin accumulated to a higher steady-state concentration than ciprofloxacin in staphylococci and higher in staphylococci than in gram-negative bacteria, confirming the data of Moreau et al. (13). Accumulation of sparfloxacin was inhibited by magnesium ions, suggesting either that sparfloxacin forms a complex which is too bulky to diffuse the cell envelope or that this agent uses a "self-promoted" accumulation pathway. A similar inhibition of accumulation by magnesium ions was effected by fleroxacin and norfloxacin (2, 10).

Sparfloxacin- and ciprofloxacin-resistant mutants were obtained at similar frequencies in all strains studied except *E. cloacae*. As with other quinolones, mutants with a decrease in susceptibility to quinolones only (*gyrA*) and mutants with decreased susceptibilities to quinolones and unrelated drugs (multiply resistant) were obtained. Previous studies have shown that *gyrA* mutations (e.g., *nfxA*, *norA*, and *cfxA*) in *E. coli* contain alleles of *gyrA* (9, 11) and multiple resistance mutations in *E. coli* (e.g., *norB*) contain alleles of *marA* and affect the expression of OmpF (6, 9). The data obtained in this study suggest that the mutants resistant to quinolones alone contain alleles of *gyrA*, since increased concentrations of quinolones are required to inhibit DNA synthesis. Our failure to show reduced expression of OmpF (or a similar OMP) in the multiply resistant mutants of *E. coli*, *E. cloacae*, and *K. pneumoniae* suggests that these mutations are not in alleles of a gene analogous to *marA*. In some but not all mutants, decreased accumulation of norfloxacin and an increase in the IC_{50} were seen. If there is a mutation decreasing the permeability of the outer membrane, the increase in IC_{50} may be an artifact, since DNA synthesis is measured in whole cells and accumulation of the radiolabeled nucleotide may be hindered. Experiments to remove the outer membrane with toluene before nucleotide was added and DNA synthesis was measured were unsuccessful. The *gyrA* gene probe was successfully inserted into mutant *K. pneumoniae* and decreased the MICs of quinolones and unrelated agents such as chloramphenicol and trimethoprim, suggesting that there was a mutation in *gyrA* which also affected the expression of unlinked genes. It is also interesting to note that some *S. aureus* mutants were also multiply resistant, suggesting a common mechanism. Decreased accumulation of sparfloxacin in *S. aureus* has been described recently (26), but no measurement of DNA synthesis or mention of cross-resistance was made. *S. aureus* expressing *norA* (decreased quinolone accumulation) showed no significant decrease in the accumulation of sparfloxacin, unlike accumulation of enoxacin (17). The decrease in the IC_{50} suggests a mutation in *gyrA*, but the presence of a permeability barrier cannot be ruled out. Fluoroquinolone resistance associated with a concomitant increase in susceptibility to nalidixic acid has recently been described for a clinical isolate of *E. coli* (12), and the phenotype appears similar to that of the mutant *S. aureus* selected in this study with ciprofloxacin.

If the recommended breakpoint concentration of sparfloxacin is similar to that of ciprofloxacin (1 μg/ml), the data obtained in this study suggest that one-step mutations can occur in *E. cloacae*, *S. marcescens*, and *K. pneumoniae* and give rise to mutants with MICs of these agents of ≥1 μg/ml. In addition, mutant *S. aureus* would be resistant to ciprofloxacin but susceptible to sparfloxacin.

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